A SIMPLE PROCEDURE FOR PREPARING DOLICHYL MONOPHOSPHATE BY THE USE OF $POCl_3$

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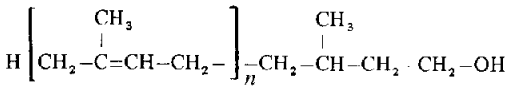
1. Introduction

In most biochemical studies on lipid-mediated transglycosylation [1] the polyprenyl phosphates are prepared by phosphorylation of naturally occuring polyprenols [2–4]. The methods of phosphorylation are adopted mainly from the field of non-enzymic synthesis of nucleotides and sugar phosphates which either give low yields of the desired polyprenyl phosphate or involve complicated multi-step procedures with elaborate phosphorylating agents. The demand for a reliable procedure for the phosphorylation of dolichol operating on a milligram or microgram scale has renewed our interest in employing $POCl_3$ as the phosphorylating agent. The use of $POCl_3$ in a trialkylphosphate solvent gained considerable advantages in phosphorylation of unprotected nucleosides [5]. The possibility of obtaining quantitative yields of phosphatidic acids from a diglyceride and $POCl_3$ on a gram scale [6] has encouraged us to design proper conditions for performing the two-step procedure without the formation of by-products in a good yield:

$$R{-}OH + POCl_3 \rightarrow R{-}O{-}POCl_2 \qquad (I)$$

$$R{-}O{-}POCl_2 + H_2O \rightarrow R{-}O{-}PO_3H_2 \qquad (II)$$

with R being various kinds of long chain dolichyls. Dolichols possess the general structure:



in which n varies from 10 (in dolichol-11) to 20 (in dolichol-21) depending upon the natural source [1].

2. Materials and methods

Dolichol-11 was prepared from plant undecaprenol isolated from leaves of *Rhus typhina* as in [7]. A mixture of dolichol-18, -19, -20 and -21 from human liver [8] was prepared as in [9] and separated into individual prenologues [10]. Tritium-labeled dolichol-19 was prepared by the $CrO_3/NaBT_4$ procedure [11]. Samples of authentic dolichyl phosphates prepared as in [3] were kindly donated by Dr W. Jankowski of our Institute.

Phosphorus oxychloride, n-hexane and triethylamine, analytical grade and precoated TLC plates of silica gel 60 (0.25 mm) were obtained from Merck, Darmstadt. Sephadex LH-20 was bought from Pharmacia, Uppsala and DEAE-cellulose (Servacel DEAE-52 for column chromatography) was a product of Serva, Heidelberg.

GDP-[^{14}C]Mannose (74 mCi/mmol) and sodium borotritide (15.3 Ci/mmol) originated from the Radiochemical Centre, Amersham. Other reagents were analytical grade from POCh, Gliwice.

For quantitative determination of total phosphate, samples containing 0.2–0.8 μg P were digested with 0.2 ml 67% perchloric acid (250°C, 10–15 min) and, after cooling and adding 0.2 ml malachite green reagent [12], the assay was performed spectrophotometrically at 660 nm. Radioactivity was measured in a Packard-TriCarb scintillation spectrometer with Bray's scintillation fluid [13]. Radioactive spots on chromatograms were localized with a TLC scanner (Berthold Instruments, Karlsruhe). For ion-exchange chromatography DEAE-cellulose (acetate form) column, 1 × 18 cm was employed. The elution of

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dolichyl phosphate was performed by the use of linear gradient of ammonium acetate (0–45 mM) in methanol [14] or in chloroform:methanol mixture (2:1, v/v). Gel filtration was performed on a 1 × 30 cm column of Sephadex LH-20, with a chloroform:methanol (2:1, v/v) mixture for elution. Solvents I, II and III for TLC were chloroform:methanol:water (65:25:4, by vol.), chloroform:methanol:15 M ammonium hydroxide (75:25:4, by vol.) and *n*-butanol:acetic acid:water (60:20:20, by vol.), respectively. The spots were detected with iodine vapors and with acid molybdate reagent [15].

3. Experimental

Preparation of dolichyl phosphate was carried out at room temperature by the use of variable amounts of dolichol and constant quantities of other reagents. Dolichols were always freshly purified by column chromatography on aluminum oxide [16]. Dolichol-11 (30 mg, 39.0 μmol) dissolved in 1 ml hexane was placed in a round bottom 5 ml flask and the solvent evaporated completely in vacuo. The residue was dissolved in 2 ml hexane and the flask capped tightly with several layers of parafilm (vessel I). In another identical flask 1 ml hexane, 20 μl $POCl_3$ (210 μmol) and 30 μl triethylamine (210 μmol) were added and a small stirring bar inserted (vessel II). The flask was capped with several layers of parafilm and stirred magnetically for 5 min. A 10 cm long polypropylene tubing 1 mm in internal diameter was sharpened on both ends, to punch through the parafilm covers on both vessels, so that the covers remained air-tight. In vessel I, containing dolichol, the end of the tubing reached the bottom of the flask, and in vessel II, containing $POCl_3$, the end of the tubing was just above the surface of the solution. A small hole was made with an injection needle first in the cover of vessel I, and then in the cover of vessel II. By applying gentle suction with a syringe mounted on the injection needle, the tubing was filled with the solution of dolichol in hexane. The needle with the syringe was then removed, and vessel I was leveled appropriately to provide slow dropping of its content by hydrostatic force into vessel II. During the transfer (15–30 min) the contents in vessel II were mixed gently by a magnetic stirrer, and after completion of the transfer mixing was continued for 15 min. The mixture was then poured into 10 ml of a mixture of acetone:water:triethylamine (88:10:2, by vol.) and left for 18 h to convert dolichyl phosphate dichloride (presumably the compound appearing in TLC in solvent I as the main spot with R_F = 0.6) into dolichyl phosphate (R_F = 0.35; spot deformed by the presence of triethylammonium chloride). The mixture was concentrated on a rotary evaporator to 1 ml aqueous milky residue. The evaporation was then repeated after the addition of 3 ml *n*-propanol. To the residue 5 ml benzene was added and the mixture evaporated. Evaporations with benzene were repeated several times until a crystalline precipitate had formed. At this stage the sample in benzene was left for 1 h and the crystalline precipitate was removed by filtration. The clear benzene solution was evaporated to dryness. The residue was dissolved in 50 ml chloroform:methanol (2:1, v/v) and applied to a DEAE-cellulose column. The elution of dolichyl phosphate with increasing concentrations of ammonium acetate in methanol (50 ml methanol in the mixing vessel and 50 ml 45 mM methanolic ammonium acetate in the reservoir) was followed by TLC in solvent I. The fractions containing dolichyl phosphate were pooled, evaporated to dryness, dissolved in 0.5 ml chloroform:methanol (2:1, v/v) and subjected to gel filtration to remove ammonium acetate. The progress of gel filtration was monitored by TLC in solvent I.

The yield of monophosphoric ester of dolichol-11 is shown in table 1. The preparation exhibited the same R_F in TLC in solvents I, II and III as the authentic compound. Both preparations were equally effective as lipid acceptors of mannose when checked with enzyme preparation from rat liver microsomes and GDP-[^{14}C]mannose [17] (the assays were kindly performed by Dr G. Palamarczyk of our Institute). The PMR spectrum of dolichyl-11 phosphate, recorded in

Table 1
Phosphorylation of dolichols with $POCl_3$

Dolichol		% Yield of dolichyl phosphate
Dolichol-11	30 mg; 39.0 μmol	72.5[a]
Dolichol-18	24 mg; 19.2 μmol	68.8[a]
Dolichol-19	35 mg; 26.6 μmol	84.0[a]
Dolichol-20	30 mg; 21.7 μmol	61.5[a]
Dolichol-21	40 mg; 27.6 μmol	69.5[a]
[^{3}H]Dolichol-19	25 μg; 19.0 nmol	56.9[b]

[a] Estimated from the weight of diammonium salt
[b] Estimated from the scintillation assay of radioactivity following TLC (fig.1)

$CDCl_3$ with a Varian EM-360 60 MHz NMR spectrometer, showed a typical pattern for dolichyl-11 residue [7]. Similar high yields of dolichyl phosphate were obtained in an identical manner with dolichol-18, -19, -20 and -21 (table 1). With the latter dolichols the purification of dolichyl phosphate on a DEAE-cellulose column was performed using the same salt gradient in chloroform:methanol (2:1, v/v) because of the insolubility of the product in methanol. The products of these syntheses were identical on TLC in solvents I, II and III with the respective authentic dolichyl phosphates. The molar proportions of the total phosphate in the preparations of dolichyl-11 phosphate, dolichyl-18 phosphate, dolichyl-19 phosphate, dolichyl-20 phosphate and dolichyl-21 phosphate were: 0.91, 0.90, 0.91, 1.00 and 1.10, respectively (theor. 1.00).

High yield of dolichyl phosphate was also obtained in identical manner with 25 μg tritiated dolichol-19 (table 1). In this experiment the milky aqueous residue resulting from incomplete evaporation of the reaction mixture treated with acetone/water/triethylamine (see above) was mixed with 10 ml benzene and 2 ml water and left for 18 h for the separation of lipid phosphates (benzene layer) from water-soluble salts. The tritium activity was present in the benzene layer. TLC of this material (fig.1) shows the presence of one main radioactive spot of R_F identical with that of authentic dolichyl-19 phosphate. There was no significant formation of other radioactive products, e.g., di- or tri-substituted phosphates of polyphosphates.

Fig.1. Scans of TLC of [^{3}H]dolichol-19 preparation (1) and of the radioactive lipids resulting from phosphorylation of [^{3}H]dolichol (2). The silica gel plate was developed in chloroform:methanol:water (65:25:4, by vol.): S, start; F, front; A and B, spots of authentic carrier dolichol-19 and dolichyl-19 monophosphate, respectively.

4. Conclusion

The successful phosphorylation of low amounts of dolichol with $POCl_3$ was due to properly chosen conditions of the reaction. We think that the simplicity and effectiveness of this method may enable wider use of dolichyl phosphates in biochemical investigations.

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